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Three-dimensional Collagen Scaffolds in Cultures of Olfactory Ensheathing Cells Used for Severed Spinal Cord Regeneration

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Abstract. *Background/Aim: The regeneration of a completely damaged spinal cord is still a challenge in modern medicine. A promising treatment method is autologous transplantation of olfactory ensheathing cells (OECs). This study aimed primarily to test methods of culturing OECs with the use of materials and reagents that are certified for pharmaceutical use in the production of an advanced cell therapy product intended for humans. Materials and Methods: The culture of OECs was performed using various modifications of the surface of the culture vessels (with fibronectin and poly-D-lysine). The number of cells was assessed after immunofluorescence staining using anti-fibronectin and anti-p75 NGF receptor antibodies. The study compared, in terms of surgical manipulations, scaffolds with OECs prepared based on 3 types of collagen: Acid Solubilized Telo Collagen and Pepsin Solubilized Atelocollagen, and the popular Corning collagen. Results: We have shown that when suspending OECs in collagen gel, it is much better to use acid-solubilized collagen (ASC) than pepsin-solubilized collagen (PSC) because the 3D collagen scaffold from ASC provides much easier handling of the product during a surgical procedure. We also found that the OEC cultures should be grown on the surface modified with fibronectin. Furthermore, we have also shown that the optimal concentration of fetal bovine serum (FBS) for culturing these cells should be around 10%. Conclusion: The culture of OECs based on reagents intended for human use can be successfully carried out, obtaining sufficient OECs content in the heterogeneous cell culture to produce a functional advanced therapy medicinal product.*

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Key Words: Olfactory ensheathing cells, acid-solubilized collagen, pepsin-solubilized collagen, spinal cord injury, spine trauma.

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The progress of science and technology has significantly improved people's health quality and life expectancy due to the increased access and effectiveness of medical therapies. One of the ongoing challenges is the achievement of functional regeneration of lesioned axons of the spinal cord in humans with tetra- or paraplegia (1-3). Over the past several decades, a great number of *in vivo* and *in vitro* experiments have allowed us to understand better the biological mechanisms regulating the regeneration processes of the nervous system after trauma. Biomaterials for the regeneration of damaged peripheral nerves have already been developed (1, 4, 5). However, research on the effective regeneration of central nervous system (CNS) axons is still mainly in preclinical research or phase I clinical trials. It is well known that the regenerative capacity of the nervous system is limited compared to other organs of the human body.

High hopes in the treatment of spinal cord injuries are associated with the use of stem cells (mostly mesenchymal stem cells) and olfactory ensheathing cells (OECs), affecting the regrowth and alignment of spinal cord axons (6-9). Medical experiments based on cell transplantation strategies are carried out to obtain functional spinal cord regeneration in cases of its total injury. New advanced therapy medicinal products (ATMPs) are developed for these purposes.

There are numerous attempts to use various types of stem cells for spinal cord injury therapy. Skin-derived precursor cells seemed to be a good solution due to the easiness of obtaining these cells from patients (10). *In vivo* studies have shown that autologous cells from mice implanted into the trauma cavity reduce it. However, an increased risk of CNS tumors has been observed long after transplantation (11, 12). Adipocyte-derived stromal cells administered together with glial cells increase the proliferation of glial cells and the elongation of axons due to the secretion of trophic factors such as NGF and BDNF (13-16). In studies of the combined application of microglia with Schwann cells, a much stronger neuroregenerative effect was observed than for each of these cell types separately. Schwann cells are particularly active in the process of repairing the nervous system—as a result of arranging themselves in columns with the so-called Bunger bands, they affect the ability of cells to differentiate, migrate, and proliferate, as well as axon myelination (17-19).

Transplantation of OECs to the area of spinal cord transection or the area of selective spinal cord lesion has been shown to restore motor and sensory function in rats (20, 21). OECs are believed to have a unique interaction with astrocytes that results in the reestablishment of the pathways for central regeneration (22). OECs have been studied not only in the context of spinal cord regeneration but also in neurodegenerative diseases, such as amyotrophic lateral sclerosis and glaucoma (23-25). These cells also have the ability to promote remyelinating damaged axons (26, 27).

So far, the first clinical trial in a patient with complete spinal cord transection, in whom autologous bulbar OECs were applied, has shown restoration of some voluntary motor functions in the lower limbs and also essential recovery of sensation. This functional recovery was mainly due to central axon regeneration, which was proved by electrophysiological and radiological studies. In this study, the glial scar in the area of spinal cord transection has been resected, followed by intraparenchymal microinjection of suspensions of OECs and ONFs. The spinal cord stumps were finally connected by autologous nerve grafts (28).

Further application of transplants of human OECs in clinical trials requires elaborating a reliable method allowing to obtain substantially high yields of bulbar OECs. Besides, it is of great importance to improve the technique of OECs delivery to the spinal cord to minimize cell loss during transplantation and to enable long-term cell survival *in vivo*. One solution to the problems mentioned above will be the application of OEC-ONF-explants instead of suspensions of cells used in the majority of preclinical and clinical studies. When preparing an OEC product in the form of an explant, it will be beneficial to use a collagen scaffold in the preparation of OECs. The product in the form of a collagen gel with cells is easier for transportation and precise placement to the target site during the surgery than the previously used cell suspension. The choice of collagen as a biomaterial for the suspension of OECs seems to be the most advantageous for several reasons. Collagen is one of the macromolecules that make up the extracellular matrix (ECM). In the ECM, collagens are present as fibrillar proteins and provide cells with structural and biochemical support (29). This material has been shown to protect axons from damage, and it has also been observed to promote neuroregeneration in peripheral nervous system damage (30).

The use of this improvement in the target medical experiment requires the selection of appropriate GMP-grade reagents certified for use in humans. Therefore, two kinds of certified type I collagen were tested in this study, one of which was acid-solubilized (ASC; acid-solubilized collagen) and the other pepsin-solubilized (PSC; pepsin-solubilized collagen). These collagens were compared with another collagen intended only for research purposes commonly used in previous animal spinal cord regeneration studies.

Materials and Methods

Surface coatings of culture vessels. The surfaces of the wells of used culture dishes—multiwell test plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and chamber slides (Nunc Lab-Tek II Chamber Slide System; Thermo Fisher Scientific, Waltham, MA, USA) were coated with either fibronectin or poly-D-lysine. The fibronectin solution (Biological Industries, Beit-Haemek, Israel) was diluted in DPBS (Gibco, Thermo Fisher Scientific) in the ratio of 1:100. Poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DPBS to the concentration of 50 μg/ml. The coating solutions were applied to the wells to cover the surface for 30 min at room temperature. The solution was then removed, and the wells were rinsed 3 times for 5 min with HBSS. The plates were then air-dried and sterilized under UV for 30 min. The surface treatment of the culture vessels was performed on the day when the cell culture was created.

Isolation of cell cultures. The olfactory bulbs were obtained post-mortem from Wistar rats weighing 250-270 g (260.7±7.6 g on average). The collected olfactory bulb was placed in a Petri dish in a PBS solution (Lonza, Basel, Switzerland) with antibiotics (Penicillin-Streptomycin; Gibco, Thermo Fisher Scientific). After blood vessels and meningeal tissue were removed under a dissecting microscope Motic SMZ-168 (Motic, Richmond, British Columbia, Canada), the weight of the collected tissue was calculated by subtraction the weights of the dish with and without the tissue. Using a scalpel, the olfactory bulb tissue was cut into smaller pieces, transferred into TrypLE solution (Gibco, Thermo Fisher Scientific) in a test tube and incubated for 15 min at 37˚C in 5% CO_2. The enzymatically digested tissue was triturated with a 1 ml plastic pipette followed by filtering through a cell strainer (Corning Inc., Corning, NY, USA) to generate a single-cell suspension. The filter was rinsed thoroughly with a culture medium—DMEM/F-12 (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; VWR International, Radnor, PA, USA) and antibiotics (Penicillin-Streptomycin; Gibco, Thermo Fisher Scientific). A total of 25 μl of the cell suspension was used to

perform the viability assay on the Countstar automated cell counter (ALIT Life Science, Shanghai, PR China). The remaining cell suspension was centrifuged for 5 min at 300×*g*, and the cells were plated at a density of 320,000 cells/well (well area 1.864 cm^2) in two types of DMEM/F-12 medium, containing 10% or 20% FBS, respectively. The cells were always seeded simultaneously on a culture plate and one well of a chamber slide (as a control culture). After 48 h, the cell confluence was checked, and it was decided whether it was necessary to add fresh medium. Five days after isolation, the medium was replaced with fresh medium. Photographs were taken at each observation to archive the progress of the cell culture process.

3D collagen scaffold. After 10 days from the isolation, collagen gels with cells were prepared. The medium was removed from the cell cultures, and the cells were washed with PBS to remove residual serum. TrypLE solution was added to the culture dish, which was then kept in an incubator at 37˚C in 5% CO_2 for 2 min to lift the cells from the surface of the wells. The cell suspension was then collected into a tube, and a medium with 10% FBS was added to neutralize the activity of TrypLE solution. Twenty-five µl of the suspension was sampled to assess cell number and viability. The remaining cell suspension was centrifuged for 5 min at 300×*g*, and then the supernatant was removed. Collagen gels containing 1.25×10^5 or 2.5×10^5 cells were prepared in two different ways. In the first method, cultures of OECs were suspended in a collagen gel according to a procedure available in the literature (7). The appropriate amount of the cell suspension was mixed with type I collagen, NaOH, and minimum essential medium (MEM; Gibco, Thermo Fisher Scientific) and deionized water intended for pharmaceutical use. The second method differed in that no additional deionized water was added to the prepared mixture. Two hundred and fifty µl of the cell suspension in collagen was transferred into the 6-well culture plate with a well diameter of 35 mm and placed in an incubator, where it polymerized to a form of a gel matrix for approximately 35-40 min (change in colour of the gel from light pink to milky). After this time, it was checked whether the gel was stable but flexible in the vertical direction. At this stage, the culture medium containing the appropriate amount of FBS, consistent with that previously used in the culture (10% or 20%), was added.

The entire process was performed with 3 different kinds of type I collagen. Two of them were of medical-grade: Acid Solubilized Telo Collagen, 6 mg/ml (Collagen Solutions, Glasgow, UK) and Pepsin Solubilized Atelocollagen, 6 mg/ml (Collagen Solutions). For comparison, the popular Corning collagen (8-11 mg/ml; Corning Inc., Corning, NY, USA), intended only for research purposes, was also used.

Viability evaluation. The viability assay was carried out on a Countstar automated cell counter (ALIT Life Science), designed to work in the GMP controlled conditions, using the trypan blue solution (Gibco, Thermo Fisher Scientific), manufactured at a cGMP-compliant facility.

Immunofluorescence procedure. Cells on chamber slides were fixed with 4% paraformaldehyde (PFA; Biotium, Fremont, CA, USA) on the day of the collagen gel preparation. After removing the culture medium, the PFA solution was added for 20 min at room temperature and discarded. The cultures were washed 3 times for 5 min with PBST (0.1% Tween 20 in PBS) and permeabilization of the cell membrane was performed using 0.1% Triton X-100 solution in PBST for 10 min at room temperature. Blocking of nonspecific binding of antibodies was performed in a solution containing 1% bovine serum albumin (BSA) in PBST for 30 min. The cell cultures were incubated with the primary antibodies anti-fibronectin (1:200; Abcam, Cambridge, UK) and anti-p75 NGF receptor (1:100; Sigma-Aldrich) in 1% BSA in PBST at 4˚C overnight. After washing off the primary antibodies thoroughly with PBST, the cultures were further incubated in the species-specific fluorescence secondary antibodies Anti-Mouse Alexa Fluor 555 (Abcam; cat. no. ab150106) and Anti-Rabbit Alexa Fluor 488 (Abcam; cat. no. ab150077) diluted at 1:500 in PBST. After rinsing in PBST, the cultures were mounted inVECTASHIELD Antifade Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and coverslipped, allowing for long-term storage of fixed and stained specimens.

Collagen scaffold stability assessment. After 48 h of incubation of cell cultures in the collagen scaffold, its stability under mechanical manipulation was checked. The possibility of detaching the collagen gel in a circular motion with a scraper and then transferring it to the transport vessel was investigated.

Statistical analysis. The data had a normal distribution and variance equality. Therefore, parametric tests were used, and data were presented as mean±standard deviation. One-way ANOVA and Fisher's LSD post-hoc analysis were performed to compare the cell number between surface modification methods (fibronectin *vs*. poly-D-lysine) and different concentrations of FBS (10% *vs*. 20%). Immunocytochemical images of the chamber slides were taken, based on which the proportion of OECs to fibroblasts in the culture was assessed using the ImageJ software. The microscopic images were taken sequentially at 14 points of the culture well of the chamber slide. Each image covered approximately 0.24 mm^2 area, thus the 14 pictures reflected 3.36 mm^2, which constituted almost 5% of the total area of a 70 mm^2 well.

Results and Discussion

It is well known that throughout life, the olfactory receptor neurons located in the nose regenerate, and their regenerating axons have the ability to enter the CNS, where they form new synaptic connections. Olfactory ensheathing cells (OECs), a type of glia, play a major role in this process. They can be isolated from the olfactory bulb or the olfactory nasal mucosa. Up to 10 times OECs can be obtained from the olfactory bulb than from the nasal mucosa (7). Collecting tissue from the nasal mucosa to obtain OECs is much less invasive than collecting the entire olfactory bulb during surgery (7, 31). However, previous studies by transplanting OECs obtained from the nasal mucosa have not shown a significant improvement in motor functions in patients (31). In comparison, under the same procedure but with OECs derived from the olfactory bulb, a significant neurological recovery was observed in a patient (28). The difference in effectiveness in promoting regeneration of CNS axons and restoring neurological

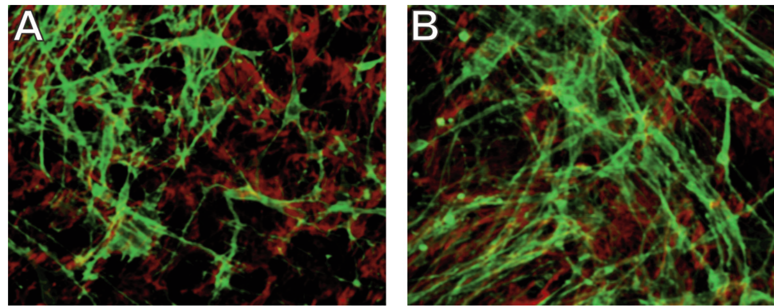


Figure 1. *Immunocytochemistry staining of the heterogeneous culture of olfactory ensheathing cells (green) and fibroblasts (red): A) cells incubated with 10% fetal bovine serum (FBS), B) cells incubated with 20% FBS.*

functions between the OECs from the olfactory bulbs and the olfactory mucosa has also been widely observed in animal research. So far, no definite answer has been worked out regarding the difference in therapy efficacy with OECs obtained from different sources (32). The most likely and obvious cause is a difference in the number of OEC in the final product and a different composition of the cells that accompany OECs in the culture—in the samples collected from the olfactory bulb, there are additionally astrocytes and neurons, and in the material from the nasal mucosa, epithelial and glandular cells as well as mesenchymal stem cells (7). In addition, OECs isolated from the olfactory bulb are characterized by upregulated gene expression related to the development of the nervous system, whereas in the case of cells from the olfactory mucosa—gene expression is related to wound healing and regulation of the extracellular matrix (33).

The team of Ying Li and Paweł Tabakow conducted a study using OECs collected from the human olfactory bulbs. They found that the number of OECs cultured from the collected samples varied in a great range. The study is still in progress, but the preliminary result suggests that preservation of tissue conditions is critical, including the donors' age, tissue retrieval techniques, tissue storing conditions, and length of time from tissue retrieval to cell culture preparation. In their study, by transplanting a pool of cells cultured from several samples, the transplant containing about 33% OECs encapsulated in collagen improved motor function in a vertical climbing test in half the rats with dorsal root injuries (34). Unfortunately, there is no specific information available on the ratio of OECs to fibroblasts sufficient for spinal cord regeneration in humans. Moreover, it cannot be ruled out that the human olfactory bulb may be a source of a lesser number of OECs compared to the rat bulb. Since the percentage of OECs in heterogeneous culture has a significant influence on the effect obtained in the patient, it is of great importance to work on methods for increasing their content in cell culture.

Previous studies have shown that the material collected from the olfactory mucosa contained about 5% of OECs. In animal studies, it was demonstrated that transplantation resulted only in a slight improvement in motor functions. Cells were unable to bridge the lesions completely and showed low migration capacity (32, 35). Minkelyte *et al.* modified the method of collecting OECs from the olfactory mucosa to increase the percentage of OECs to at least 20% in a heterogeneous culture. Transplantation of this improved culture of mucosal OECs showed a clear improvement in motor activity (7). This result is promising and encouraging, as previous studies have suggested that 33-50% of OECs are sufficient to allow spinal cord regeneration after transplantation (34, 35).

Our study assessed cell morphology in heterogeneous cultures of OECs and fibroblasts. On the day the cell culture was transferred to the collagen scaffold, the ratios of OECs to fibroblasts for the individual experiments performed were 48:52, 54:46, and 40:60. We obtained an average of 47.3% OECs in a heterogeneous culture with fibroblasts. According to previous studies, these proportions of OECs and fibroblasts should be sufficient to produce an effective biological product. The ratio of OECs to fibroblasts was determined in the chamber slides, which will be used as the control culture for the quality evaluation during the development of the biological product (Figure 1). By assessing the morphology of the cells without immunostaining, it is virtually impossible to determine the number of OECs and fibroblasts. Only after staining with antibodies characteristic of a given phenotype of cells isolated from the olfactory bulb can the number of cells of a particular type be assessed. Seeding the cells on a small surface area of the chamber slides ensures the least possible loss of cells needed to manufacture the product and, at the same time, provides the necessary control for quality assessment.

In cultures of cells obtained from both the olfactory bulb and the olfactory mucosa, in addition to OECs, there are mainly olfactory nerve fibroblasts (ONFs). Fibroblasts are easy to isolate, and therefore their content in the obtained

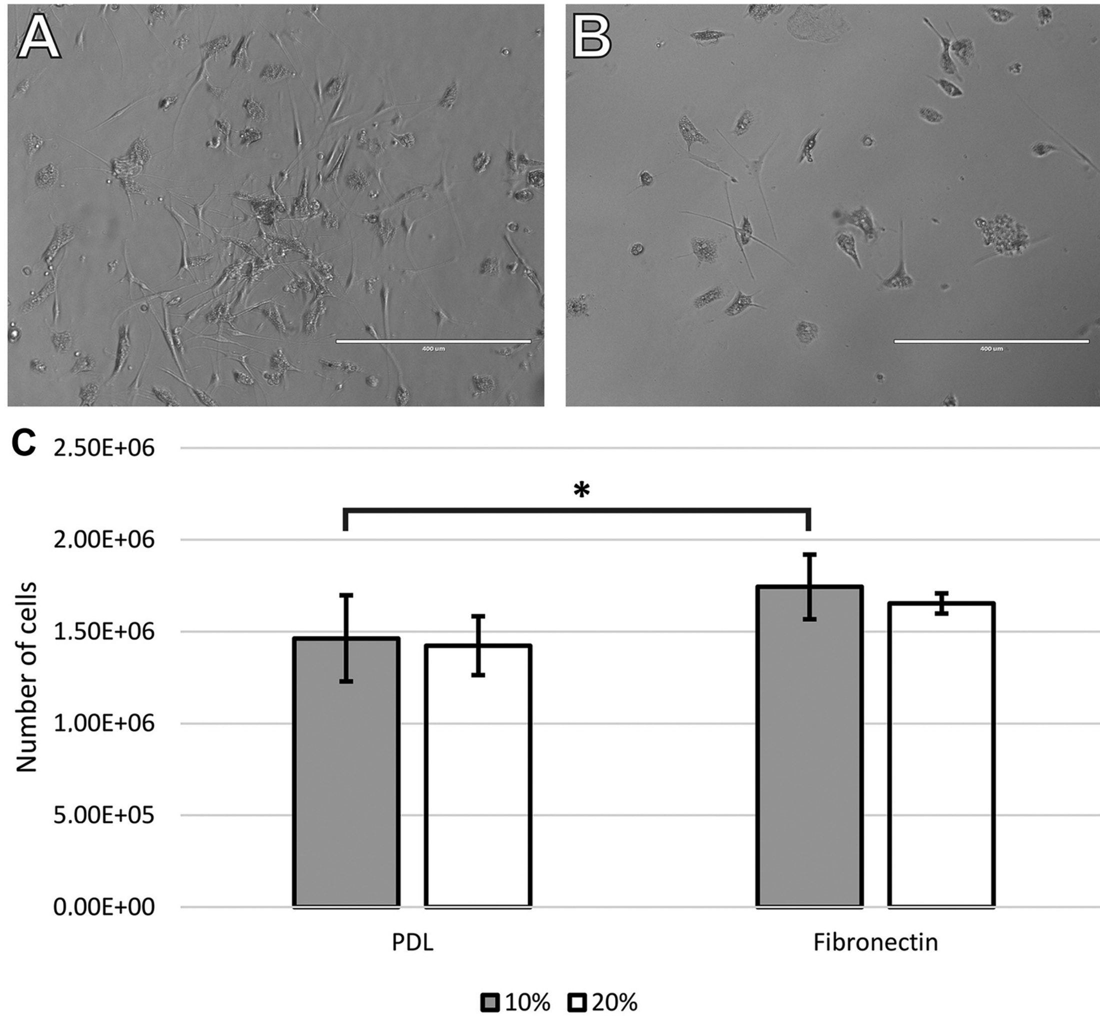


Figure 2. *Cell culture four days after isolation: A) cells grown in plates with the fibronectin-modified surface; B) cells grown in plates with the surface modified with poly-D-lysine; C) number of cells with different surface modifications and with different content of fetal bovine serum (FBS) in culture medium.*

cultures is always significant (33). The substances they release can provide many beneficial trophic factors for the remaining isolated cells. Therefore, ONFs are good support for OECs in cell cultures. However, when the culturing period is too long, a decrease in the ratio of OECs to ONFs is observed. Therefore, the cultivation can be carried out optimally only for up to two weeks. In order to accelerate the achievement of cell confluence, it is worth modifying the surface of the culture vessels by applying an appropriate coating. Fibronectin and poly-D-lysine (PDL) were used as coating substances since they were currently available with the relevant GMP certificates. Poly-D-lysine only facilitates cell adhesion (36). Numerous studies have shown a positive effect of fibronectin on the morphology and migration of nerve cells (37, 38). With the use of 10% FBS, a significantly greater (p=0.046) number

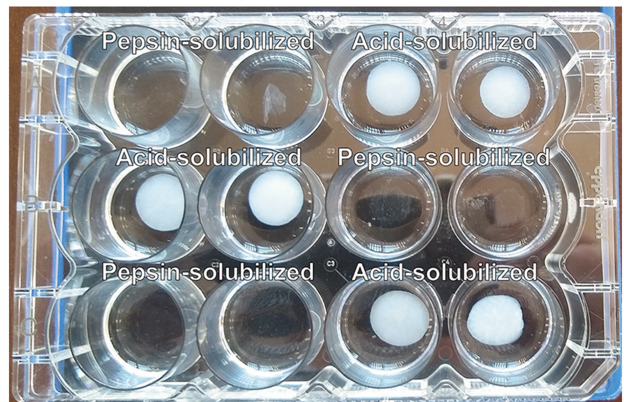


Figure 3. *Collagen gel with olfactory ensheathing cells after 35-40 min polymerization.*

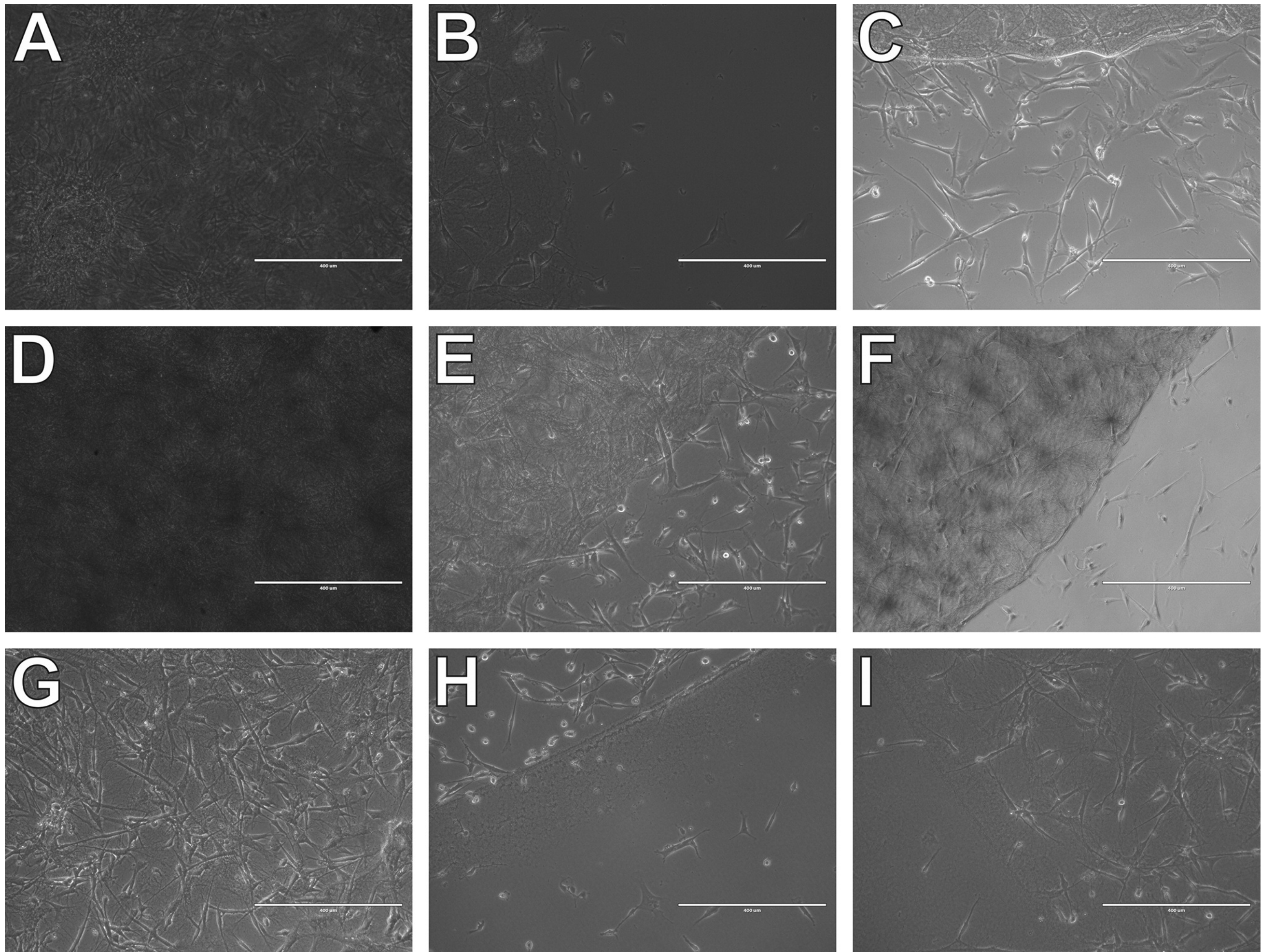
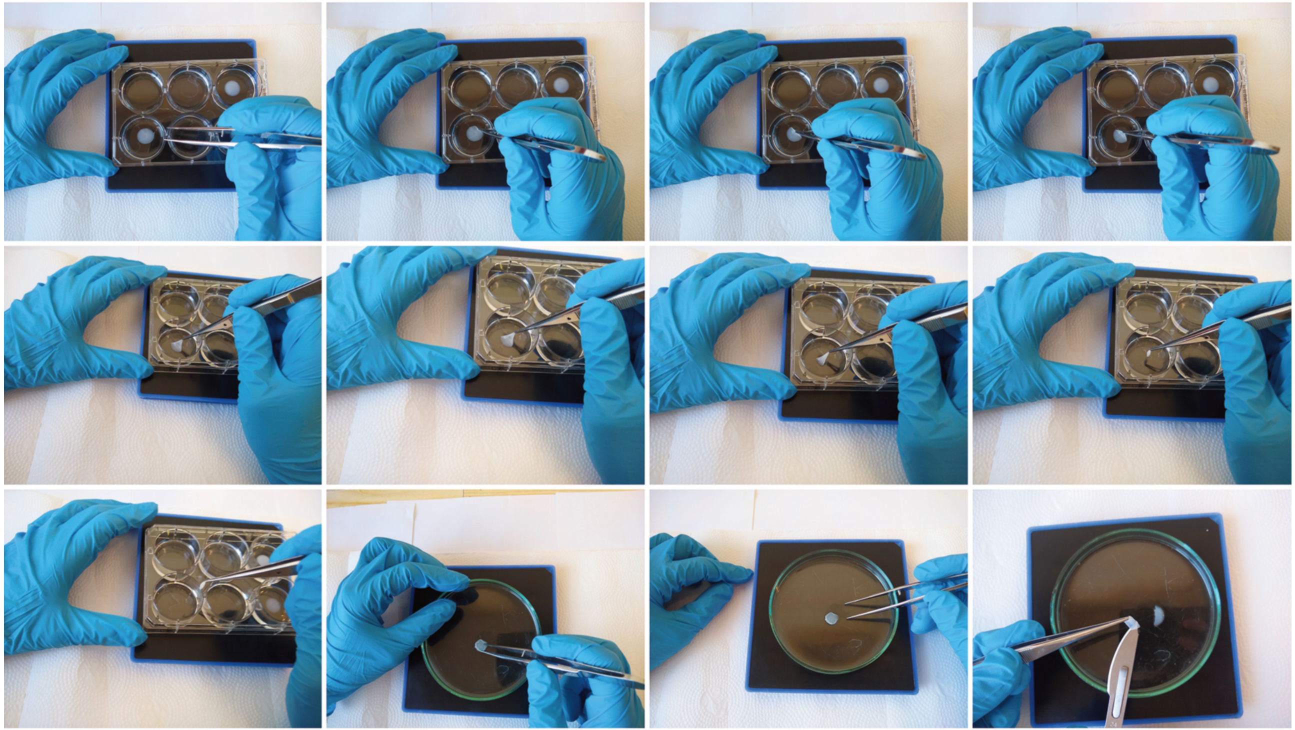


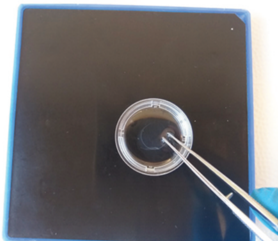
Figure 4. *Cells in collagen scaffold: A-C) Corning collagen, D-F) acid-solubilized collagen, G-I) pepsin-solubilized collagen; A, D, G) first preparation method with 2.5×10^5 cells; B, E, H) first preparation method with 1.25×10^5 cells; C, F, I – second preparation method with 1.25×10^5 cells.*

of cells was obtained in our study on the surfaces modified with fibronectin (1.74×10^6±55,075) compared to those with PDL (1.46×10^6±160,104) (Figure 2C). Therefore, surface modification using fibronectin was selected for culturing the cells to prepare the biological product. As can be seen, the cultures of OECs on the surface of the fibronectin-coated wells (Figure 2A) on day 4 from isolation reached a higher confluence than that on PDL (Figure 2B).

The study also examined the effect of the enzyme used for detaching cells from the surface of the culture dish. It was necessary to replace the traditionally used trypsin with another non-animal origin reagent. CTS TrypLE Select Enzyme certified for manufacturing cell, gene, or tissue-based products was used, reducing viral or prion contamination risk (39). A cell viability assay was carried out, which confirmed that the enzyme did not have a negative effect on the culture condition—after a 2-minute treatment, viability was 97.5±2.1%.

In CNS injuries, cell transplantation is mainly performed by microinjections in many places at different depths (28, 31, 40, 41). However, this process is time-consuming and imprecise (it is difficult to retain cells at the target site) and is also associated with the risk of cell loss during the process of their preparation and transplantation. A possible solution of this problem may be to change the form of cell administration in subsequent patients into a collagen gel seeded with cells. This improvement is mainly aimed at facilitating operational manipulations. The use of collagen gel should also accelerate the regeneration processes of the spinal cord, even in the case of obtaining cell cultures with a ratio of OECs to fibroblasts below 1:1. Collagen occurs naturally in the extracellular space and should facilitate the





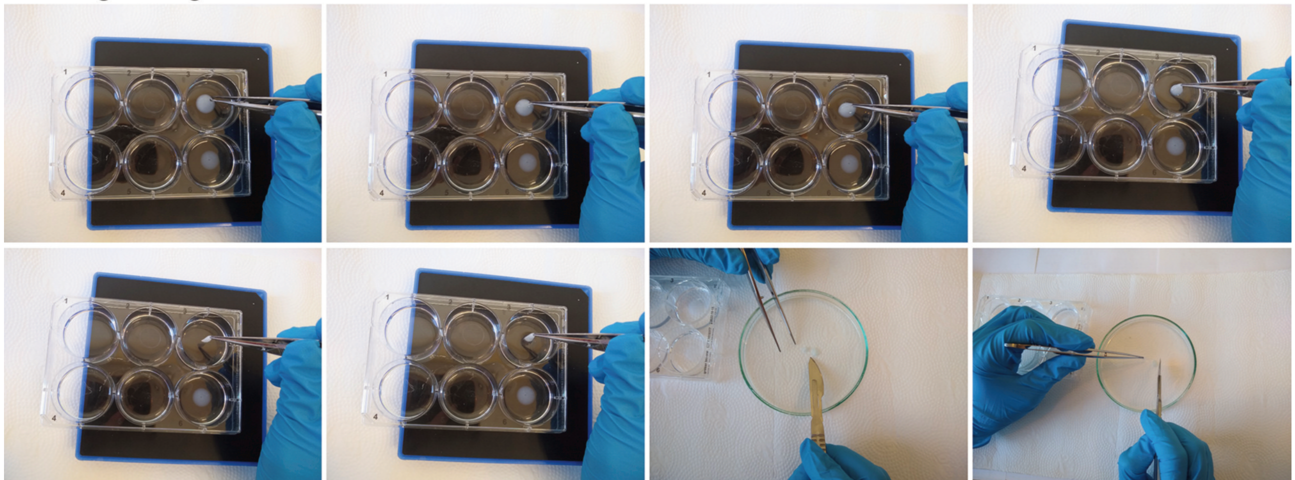


Figure 5. *Detaching collagen gel from the surface of multiwell plates*.

growth of nerve cells. Corning collagen is widely used in laboratory research on spinal cord regeneration but is not certified for use in products for human application. We tested two GMP-grade collagens—acid-solubilized (ASC) and pepsin-solubilized (PSC). Comparing macroscopically the scaffolds formed from these three kinds of collagen, the structure of the tested ASC resembles Corning collagen (which is also acid-solubilized). PSC created a less compact structure without a clear border between the scaffold and the rest of the surface of the well (Figure 3). The cultures in the scaffolds made of tested ASC and Corning collagen are compact multi-layered (Figure 4A and D), whereas in the PSC scaffold, the cells were only observed in 2-3 layers (Figure 4G).

After 48 h of culturing the cells in the collagen scaffold, the structure was assessed for product manipulation. When ASC or Corning collagens were used, the products were detached from the well surface (in a circular motion with a scraper) without disturbing the structure (Figure 5). In contrast, it was impossible to keep the structure intact with PSC—the edges were frayed. The transfer from the culture dish to the transport vessel (in which it will be transferred to the surgical theatre) will be much easier in the case of the ASC collagen.

An important aspect of this study was also the assessment of the number of cells most beneficial for the preparation of the product and the use of water or no water in the process of developing the collagen scaffold. It was observed that after 48 h of incubation using 1.25×10^5 cells, the structure was much less compact and with fewer layers than with 2.5×10^5 cells. At the same time, it did not have a negative impact on the manipulation processes, such as detaching from the surface of the well and transferring the product to another culture vessel. According to protocols available in the literature, water is commonly used to prepare 3D scaffolds with cell cultures. Water is common in cell culture—it is the main component of buffers and media, but an excess can also negatively affect cells. Therefore, a commonly used protocol has been slightly modified to determine whether it is possible to prepare a 3D scaffold without water. Comparing the obtained scaffolds for each of the tested collagens, no differences were found between the obtained products depending on the use of water in the preparation process (Figure 4B, C, E and F).

Bearing in mind the need to select GMP-grade reagents for the next biological product transplantation in a patient with spinal cord injury, as part of this study, we were able to test all the procedures related to the process of isolation and encapsulation of cells in collagen gel. All the reagents and plastics used in this study were certified for use in humans. Only the use of serum for cell culture is a potential risk. However, at this stage, it cannot be avoided. The available studies comparing the effect of lowering the serum content in the culture medium from 10% to 2% showed that in the cocultures of OECs and ONFs treated with the medium with a reduced amount of FBS, a higher percentage of OECs was observed compared to fibroblasts. However, the total number of OECs was significantly lower than that in cultures incubated with 10% FBS (42). The observed relationship regarding the FBS content in the culture medium was independent of the surface modification method (poly-D-lysine or laminin). The comparison of the total number of cells obtained at the end of the culture in our study, as well as the counting performed during the viability assay, show that there is no significant difference between using the medium supplemented with 10% and 20% serum (p=0.44 for cells grown on a surface modified with fibronectin; p=0.82 for the surface modified with PDL).

In conclusion, prior to future cellular product transplantation in patients with a complete spinal cord injury, it is advisable to use acid-solubilized collagen (ASC) in the process of preparing the olfactory ensheathing cells. The collagen scaffold can be prepared with or without water—this is irrelevant to the quality of the final cellular product. The thickness and number of cell layers in the collagen scaffold depend mainly on the number of cells previously collected during isolation from the explant.

Heterogeneous cultures of olfactory ensheathing cells with fibroblasts should be grown on fibronectin-coated plates in a medium supplemented with 10% FBS. In addition, it was confirmed that the TrypLE enzyme could be used successfully in the cell culture process instead of the animal-derived trypsin.

Conflicts of Interest

The Authors declare that they have no competing interest in relation to this study.

Authors' Contributions

Conceptualization, W.F., B.W. and D.L.; methodology, W.F., B.W., Y.L and D.L.; formal analysis, B.W.; investigation, W.F., B.W., P.J. and A.K.-K.; data curation, B.W.; writing—original draft preparation, B.W.; writing—review and editing, B.W., D.L. and P.T.; visualization, B.W. and P.J.; supervision, Y.L. and P.T.; project administration, W.F. and B.W.; funding acquisition, W.F. All Authors have read and agreed to the published version of the manuscript.

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